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(54) Title: FUSION PROTEINS OF INTERFERON ALPHA MUTEINS WITH IMPROVED PROPERTIES

(57) Abstract: The invention concerns human interferon alpha and in particular modified forms of interferon alpha 2 with improved properties. The improved proteins contain amino acid substitutions at specific positions that confer increased relative activity in biological assays. The invention provides also modified interferon alpha with improved biological activity concomitant with reduced immunogenic potential in the protein. The improved proteins are intended for therapeutic use in the treatment of diseases in humans.

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FUSION PROTEINS OF INTERFERON ALPHA MUTEINS WITH IMPROVED PROPERTIES

FIELD

5 The invention concerns human interferon alpha and in particular modified forms of interferon alpha 2 with improved properties. The improved proteins contain amino acid substitutions at specific positions that confer increased relative activity in biological assays. The invention provides also modified interferon alpha with improved biological activity concomitant with reduced immunogenic potential in the protein. The improved
10 proteins are intended for therapeutic use in the treatment of diseases in humans.

BACKGROUND

Interferon alpha 2 (IFN α 2) is an important glycoprotein cytokine expressed by activated macrophages. The protein has considerable clinical importance as a broad spectrum anti-
15 viral, anti-proliferative and immunomodulating agent. Recombinant and other preparations of IFN α 2 have been used therapeutically in a variety of cancer and anti-viral indications in man [reviewed in Sen, G.G. and Lengyel P, (1992), *J. Biol. Chem.* 267: 5017-5020]. Currently there are a number of IFN α preparations in clinical use, including native recombinant IFN α 2s produced in *E. coli* (IFN α 2a, RoferonA[®], Hoffman-La
20 Roche; IFN α 2b, IntronA[®], Schering-Plough; IFN α 2c, Berofor[®], Basotherm) and more recently a synthetic IFN α , also produced in *E. coli*, based upon the consensus sequence of all subtypes (Infergen[®], InterMune).

A major use of IFN α 2 is the treatment of chronic hepatitis C virus (HCV) infection.
25 Treatment with IFN α alone results in sustained virus clearance in around 10% of patients, although more recently sustained viral responses of 40% have been achieved with the combination of IFN α 2 with ribavirin [Davis GL, et al, (1998) *N. Engl. J. Med.*; 339:1493-1499; McHutchison JG et al (1998) *N. Engl. J. Med.*; 339:1485-1492; Reichard O, et al (1998) *Lancet.* 351:83-87]. IFN α therapy is intensive and associated with severe side
30 effects leading to withdrawal of treatment in up to 20% of cases. The rationale for intensive therapy is that IFN α 2b has a relatively short serum half-life [Glue P, et al (2000) *Clin. Pharmacol. Ther.*; 68:556-567], requiring administration by subcutaneous injection once daily or three times weekly for anti-viral efficacy.

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The short half-life and frequent dosing have been recognised as problematic in long-term treatment. To address this 'pegylated' versions of RoferonA[®] and IntronA[®] (Pegasys[®] and Peg-Intron[®]) have been introduced and a similar version of Infergen[®] is in phase II clinical trials. These modified interferons are conjugated to polyethylene glycol moieties
5 which increases the serum half-life 10 to 20 fold (6,7), thereby reducing the dosing frequency to once weekly (180µg or 1.4µg/Kg for Peg-Intron[™] and Pegasys[™] respectively) without adversely affecting clinical efficacy [Glue P. et al (2000) *ibid*; Perry CM, et al (2001) *Drugs* ;61:2263-2288; Glue P, et al (2000) *Hepatology*;32:647-653]. In these studies, the side effect profiles are similar to unmodified interferon.

10

Another strategy for increasing serum half-life is to link IFNα to human serum albumin [Osborn BL, et al (2002) *J. Pharmacol. Exp. Ther.*;303:540-548]. Albuferon[®] consists of IFNα linked to the C-terminus of human serum albumin and, in cynomolgus monkeys, has a half-life 3 fold greater than that of pegylated IFNα and 18 fold greater than
15 unmodified IFNα. Data from studies in humans are not yet available for this molecule. However for both pegylated and albumin linked IFNα, the *in vitro* specific activity of the modified proteins is reduced compared to native protein, to 28% with Peg-Intron[®] [Grace M, et al. (2001) *Cytokine Res.*; 21:1103-1115] and to 10% or less with Pegasys[®] and Albuferon[®] [Osborn BL, et al (2002) *ibid*; Bailon P, et al (2001). *Bioconjug Chem.*
20 12:195-202].

Despite the significant therapeutic benefit found in using IFNα, resistance to therapy in certain patients has been documented and one important mechanism of resistance has been shown to be the development of neutralising antibodies detectable in the serum of
25 treated patients [Quesada, J.R. et al (1985) *J.Clin. Oncology* 3:1522-1528; Stein R.G. et al (1988) *ibid*; Russo, D. et al (1996) *Br. J. Haematol.*;94:300-305; Brooks M.G. et al (1989) *Gut* 30: 1116-1122]. An immune response in these patients is mounted to the therapeutic interferon despite the fact that a molecule of at least identical primary structure is produced endogenously in man. Repeated dosing over several months
30 induces anti-IFNα neutralising antibodies in up to 80% of patients, depending upon the indication [Schellekens H, et al (1997) *J Interferon Cytokine Res.* 17 Suppl 1:S5-8], with the reported frequency for chronic HCV infection ranging from 7% to 60% [Schellekens H, et al (1997) *ibid*]. Available evidence suggests that patients who develop neutralising

antibodies are more likely to fail to respond to treatment and suffer relapse than those who do not develop antibodies [Ross C, et al (2002) *J Interferon Cytokine Res.*; 22: 421-426; McKenna R. M, et al (1997) *J. Interferon Cytokine Res.*; 17:141-143; Russo D, et al (1996) *ibid*; Milella M, et al (1993) *Liver*; 13:146-150; Primmer O. (1993) *Cancer* ;
5 71:1828-1834], although in some cases treatment can be rescued by the subsequent use of purified leukocyte interferon [Russo D, et al (1996) *ibid*; Oberg K, & Alm G. (1997) *Biotherapy*; 10:1-5; Tefferi A, & Grendahl D. C. (1996) *Am. J. Hematol.* ;52: 231-233; Milella M, et al (1995) *Hepatogastroenterology*; 42:201-204].

10 The reason for the development of antibodies to recombinant IFN α is unclear since the protein is present naturally and expression increases sporadically in response to events such as viral infection. The route and frequency of dosing, the immune modulatory effects of IFN α , and the presence of protein aggregates in the pharmaceutical preparations may all play a role in the breakdown of immune tolerance. However,
15 irrespective of any facilitating factors, the pivotal feature leading to the induction of an immune response is the presence within the protein of peptides that can stimulate the activity of T-cells via presentation on MHC class II molecules. Such peptide sequences are "T-cell epitopes" and are commonly defined as any amino acid residue sequence with the ability to bind to MHC Class II molecules. Implicitly, a "T-cell epitope" means an
20 epitope which when bound to MHC molecules can be recognised by a T-cell receptor (TCR), and which can, at least in principle, cause the activation of these T-cells by engaging a TCR to promote a T-cell response.

From the foregoing there is clearly a continued need for IFN α 2 analogues with enhanced properties. Desired enhancements include alternative schemes and modalities for the
25 expression and purification of the therapeutic, but also and especially, improvements in the biological properties of the protein. There is a particular need for enhancement of the *in vivo* characteristics when administered to the human subject. In this regard, it is highly desired to provide IFN α 2 with reduced or absent potential to induce an immune response and enhanced biological potency in the human subject.

30

The inventors have previously disclosed the critical regions of the IFN α 2 molecule comprising the T-cell epitopes driving the immune responses to this autologous protein and have provided compositions that reduce the effectiveness or wholly eliminate these

sequences from being able to act as T-cell epitopes [WO 02/085941]. Such compositions have been achieved by alteration of the amino acid sequence of the IFN α 2 protein, for example by substitution, and the present invention is concerned also with IFN α 2 molecules in which amino acid substitution and or combinations of substitution have been
5 conducted. However in the present case, new substitutions and combinations of substitutions made confer the surprising property of significantly enhancing the biological activity of the molecule and such an enhancement in combination with substitutions achieving a reduced immunogenic profile for the protein provide for an improved IFN α 2 molecule.

10 Others have provided modified IFN α 2 and methods of use and include for example US, 4,496,537; US,5,972,331; US,5,480,640; US,5,190,751; US, 4,959,210; US, 5,609,868; US, 5,028,422 and others.

US, 5,723,125 describes a fusion protein comprising wild-type human IFN α joined via a peptide linker to a human immunoglobulin Fc fragment. The IFN α domain is oriented N-
15 terminal to the Fc domain in the claimed fusion protein.

US, 6,204,022 describes IFN α analogues bearing substitutions from WT especially at positions 19, 20, 22, 24 and 27 and characterised by reduced cytotoxicity in a biological assay.

The general category of "human Fc fusion proteins" and suitable vectors for their
20 production have been described previously [US, 5,541,087; US, 5,726,044 Lo et al (1998), *Protein Engineering* 11:495 - 500].

SUMMARY OF THE INVENTION

The invention provides human interferon alpha 2 molecules containing amino acid
25 substitutions. The amino acid substitutions confer improved properties to the protein. The improved properties concern the specific biological activity of the protein and also the immunogenic properties of the protein.

The molecules of the invention are fusion proteins comprising a human immunoglobulin
30 constant region moiety linked with a human IFN α mutein.

The molecules of the invention have new and inventive properties. Such molecules may cause benefit for a patient suffering from a disease especially suffering from chronic hepatitis C virus infection.

- 5 The molecules of the invention are characterised by the protein sequences defined herein as SEQ ID Nos 2 – 22.

The molecules of the invention are further characterised by a relative activity in a signalling assay of between greater than 1.3 and 10 fold. In some embodiments the relative activity in a signalling assay is 2 fold or 3 fold or 5 fold or 7 fold or 10 fold or 17
10 fold.

The molecules of the invention are further characterised yet still by a relative activity in an anti-viral assay of between 2 and 36 fold. In some embodiments the relative activity in an anti-viral assay is 2 fold or greater, or is 3 fold or greater, or is 4 fold or greater, or is 7
15 fold or greater or is about 36 fold.

A most preferred molecule of the invention is characterised by the protein sequence SEQ ID No 2 and is further characterised by a relative activity of greater than 9 fold in a signalling assay and about 36 fold in an anti-viral assay and about 1 in an anti-
20 proliferation assay.

A further preferred molecule of the invention is characterised by the protein sequence SEQ ID No 3 and is further characterised by a relative activity of greater than 1.3 fold in a signalling assay and about 7.4 fold in an anti-viral assay and about 1 in an anti-proliferation assay.

- 25 The molecules of the invention may be further characterised still by activity in an anti-proliferation assay of between 13 and 16 pg (picogram) interferon- α per ml (millilitre). The most preferred molecules of the invention are characterised yet further still by comprising sequences demonstrated to show reduced immunogenicity in human cells.

- 30 In summary the invention is concerned with the following issues:

- A modified interferon alpha 2 molecule having the biological activity of human interferon alpha 2 containing one or more amino acid substitutions;
- a modified interferon alpha 2 molecule having the biological activity of human interferon alpha 2 and comprising a human immunoglobulin constant region (Fc)

domain and containing one or more amino acid substitutions within the interferon alpha 2 domain as specified above or below;

- a modified interferon alpha 2 molecule having the biological activity of human interferon alpha 2 and comprising a human Fc domain and containing one or more amino acid substitutions within the interferon alpha domain and being further characterised by demonstrating reduced immunogenicity to humans especially in comparison to interferon alpha molecules not containing the amino acid substitutions of the invention;
- a general method for the recovery of IFN α muteins with improved properties involving;
 - a) identification of T-cell epitopes;
 - b) conducting single amino acid substitutions within T-cell epitope regions and selecting functionally active muteins;
 - c) optionally, conducting fine mapping studies of critical residues involved in T-cell activation and testing double, or triple of more substitutions for immunogenicity;
 - d) selecting individual muteins with the most favoured function and immunogenicity profile for constitution as multiply substituted muteins and function testing said same new proteins;
 - e) testing functionally active multiply substituted mutein sequences for reduced immunogenicity using time course immunogenicity assays;

- a modified interferon alpha 2 molecule of structure:

X⁰-CDLPQTHSLGSRRTLMLLAQMRRX¹SLFSLKDRHDFGFPQEFGNQFQKA
 ETIPVLX²EMX³QQIX⁴NLFSTKDSSAAX⁵DETL LDKFYTELX⁶QQLNDLEACVI
 QGVGVTTETPLMKEDSX⁷LAVRKYFQRITX⁸YLKEKKYSPCAWEVVRAEIMRS

FSLSTX⁹LQESLRSKE,

whereby

X⁰ = is Fc or Fc-Linker,

Fc = an Fc domain of an antibody

Linker = a linker peptide consisting of 6 to 25 amino acids

X¹ = I, Q

X² = H, Y

X³ = I, T;

X⁴ = F, T, A

X⁵ = W, H;

- 7 -

$X^6 = Y, D;$

$X^7 = I, N, T, P, R$

$X^8 = L, T, H, D, S, N$ and

$X^9 = N, S;$

5 with the provision that an IFN α molecule wherein simultaneously

$X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = W, X^6 = Y, X^7 = I, X^8 = L$ and $X^9 = N$

is excluded. In other words: the provision excludes the wild-type IFN fusion proteins.

In particular the invention relates to

- An IFN α 2 mutein, wherein
- 10 $X^5 = H$ and $X^8 = N$.
- An IFN α 2 mutein, wherein
- $X^3 = T$ and $X^4 = A$.
- An IFN α 2 mutein, selecting from the group consisting of the following compounds:
 - (i) $X^1 = Q, X^2 = H, X^3 = T, X^4 = A, X^5 = H, X^6 = Y, X^7 = R, X^8 = N$ and $X^9 = N$
 - 15 (ii) $X^1 = Q, X^2 = H, X^3 = I, X^4 = F, X^5 = H, X^6 = Y, X^7 = I, X^8 = L$ and $X^9 = N$
 - (iii) $X^1 = I, X^2 = H, X^3 = T, X^4 = A, X^5 = H, X^6 = Y, X^7 = T, R$ or $N, X^8 = N$ and $X^9 = N$
 - (iv) $X^1 = I, X^2 = H, X^3 = T, X^4 = A, X^5 = H, X^6 = Y, X^7 = I, X^8 = L$ and $X^9 = N$
 - (v) $X^1 = I, X^2 = Y, X^3 = I, X^4 = T, X^5 = H, X^6 = Y, X^7 = I, X^8 = L$ and $X^9 = N$
 - (vi) $X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = H, X^6 = Y, X^7 = P, T$ or $N, X^8 = L$ and $X^9 = N$
 - 20 (vii) $X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = H, X^6 = Y, X^7 = I, X^8 = T$ and $X^9 = S$
 - (viii) $X^1 = I, X^2 = H, X^3 = T, X^4 = F, X^5 = H, X^6 = Y, X^7 = I, X^8 = T$ and $X^9 = S$
 - (ix) $X^1 = I, X^2 = H, X^3 = T, X^4 = F, X^5 = W, X^6 = Y, X^7 = I, X^8 = T$ and $X^9 = S$
 - (x) $X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = W, X^6 = Y, X^7 = I, X^8 = T, S, N, H$ or D and
 - $X^9 = N$
 - 25 (xi) $X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = H, X^6 = Y, X^7 = I, X^8 = L$ and $X^9 = N$
 - (xii) $X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = W, X^6 = D, X^7 = I, X^8 = L$ and $X^9 = N$
 - An IFN α 2 mutein, wherein Fc is a human immunoglobulin heavy chain constant region domain, which is linked by its C-terminus to said mutein.
 - An IFN α 2 mutein, wherein the Fc domain is a monomer.
 - 30 • An IFN α 2 mutein, wherein the linker peptide consists of 12 to 20 amino acids.
 - An IFN α 2 mutein, wherein the linker peptide is (G)4S(G4)S(G4) SG

For the avoidance of doubt, the particularly advantageous muteins of IFN α 2 and which are each embodiments of the invention are characterised according to the details of Fig. 1.

The mutant proteins of the present invention are readily made using recombinant DNA techniques well known in the art and the invention provides methods for the recombinant production of such molecules.

- 5 In as far as this invention relates to modified $\text{INF}\alpha 2$, compositions containing such modified $\text{INF}\alpha 2$ proteins or fragments of modified $\text{INF}\alpha 2$ proteins and related compositions should be considered within the scope of the invention. In another aspect, the present invention relates to nucleic acids encoding modified $\text{INF}\alpha 2$ entities. In a further aspect the present invention relates to methods for therapeutic treatment of
- 10 humans using the modified $\text{INF}\alpha 2$ proteins.

DETAILED DESCRIPTION OF THE INVENTION

- In nature, the mature $\text{INF}\alpha 2$ protein is single polypeptide of 165 amino acids. Several different subtypes of human $\text{INF}\alpha 2$ are known, each showing minor differences between
- 15 primary amino acid sequences. Thus $\text{INF}\alpha 2a$ and $\text{INF}\alpha 2b$ differ in only one residue at position 23 of the mature protein chain being lysine in $\text{INF}\alpha 2a$ and arginine in $\text{INF}\alpha 2b$. Whilst the disclosures of the present invention are directed towards the sequence of $\text{INF}\alpha 2b$, it can be seen that for all practical purposes the sequence of $\text{INF}\alpha 2a$ may be considered interchangeably with the subject $\text{INF}\alpha 2b$ subtype of the present invention.
- 20 The amino acid sequence of $\text{INF}\alpha 2b$ (depicted as single-letter code) is as follows:

CDLPQTHSLGSRRTLMLLAQMRRISLFSCCLKDRHDFGFPQEEFGNQFQKAETIPVLHEMI
QQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNLEACVIOGVGVTTETPLMKEDSILAVR
KYFQRITLYLKEKKYSPCAWEVVRAEIMRSFSLSTNLQESLRSKE

25

The term " $\text{IFN}\alpha$ " is used herein to denote human interferon alpha 2. In some instances the term is also used more broadly herein to include fusion proteins (see below) comprising an interferon alpha moiety and or more especially an interferon alpha mutein.

- 30 The term "mutein" is used herein to denote an $\text{IFN}\alpha$ protein engineered to contain one or more amino acid substitutions differing from the above native sequence.

The term "peptide" as used herein, is a compound that includes two or more amino acids. The amino acids are linked together by a peptide bond.

A peptide bond is the sole covalent linkage between amino acids in the linear backbone structure of all peptides, polypeptides or proteins. The peptide bond is a covalent bond, planar in structure and chemically constitutes a substituted amide. An "amide" is any of a group of organic compounds containing the grouping -CONH-.

There are 20 different naturally occurring amino acids involved in the biological production of peptides, and any number of them may be linked in any order to form a peptide chain or ring. The naturally occurring amino acids employed in the biological production of peptides all have the L-configuration. Synthetic peptides can be prepared employing conventional synthetic methods, utilizing L-amino acids, D-amino acids, or various combinations of amino acids of the two different configurations. Some peptides contain only a few amino acid units. Short peptides, e.g., having less than ten amino acid units, are sometimes referred to as "oligopeptides". Other peptides contain a large number of amino acid residues, e.g. up to 100 or more, and are referred to as "polypeptides". By convention, a "polypeptide" may be considered as any peptide chain containing three or more amino acids, whereas a "oligopeptide" is usually considered as a particular type of "short" polypeptide. Thus, as used herein, it is understood that any reference to a "polypeptide" also includes an oligopeptide. Further, any reference to a "peptide" includes polypeptides, oligopeptides, and proteins. Each different arrangement of amino acids forms different polypeptides or proteins. The number of polypeptides—and hence the number of different proteins—that can be formed is practically unlimited.

Since the peptide bond is the sole linkage between amino acids, all peptides, polypeptides or proteins have defined termini conventionally referred to as the "N-terminus" or "N-terminal" residue and the "C-terminus" or "C-terminal residue". The N-terminal residue bears a free amino group, whereas the C-terminal residue bears a free carboxyl group.

All sequences of consecutive amino acids accordingly have an orientation N-terminal to C-terminal. Where fusion proteins are constituted or differing domains are connected within a protein species their relative orientation may be described as "N-terminal" or "C-terminal".

The term "fusion protein" is used herein to refer to a protein molecule comprising two or more functionally distinct protein domains within a single polypeptide chain. The protein moieties in the fusion protein may be directly coupled or may be joined via a linker peptide.

5

A "linker" or "linker peptide" refers herein to a peptide segment joining two moieties of fusion protein. Linker peptides suitable for this invention include peptide having 5 to 25 amino acids, preferably 10 to 20 amino acids, more preferably 15 – 20 amino acids. An example of a linker peptide is provided by the generic formula $((G)_4S)_xG$, wherein x is 1, 2, 3 or 4. The linker peptide preferred according to the present invention is $(G)_4S(G)_4S(G)_4SG$. However also other linker peptides of the prior art which have more than 10 amino acids are preferably suitable.

US,5,723,125 claims a hybrid interferon molecule of type IFN-L-Fc (where L= linker) wherein the linker sequence is GSGSGSGGGSGGGGS. The said linker is 16 residues and is considered "comparatively long". The above linker is a variant of the well known peptide linker $(GGGGS)_3$ described by Huston et al [Huston, et al (1988) *Proc Natl. Acad. Sci. USA* 85:5879]. Further shorter sequence variants of this linker are also known, such as $(GGGGS)_n$ wherein n = 1, 2 or 3 to give a linker of 5, 10 or 15 residues [Holliger, P. et al (1993) *Proc Natl. Acad. Sci. USA* 90:6444]. A particularly short version of this linker comprising 4 residues (GGGG) has been used in US, 6,686,179.

Other examples of peptide linkers recognised in the art include all of the following:
(A)₃, (A)₄, (A)₅, GG, GS, GGG, (G)₇, GPG, GPGG, EFGGGGGTA.

Fusion proteins are commonly produced by means of recombinant DNA techniques and as such can be considered artificial proteins having no direct counterparts in nature (natural fusion proteins can arise, for example via chromosomal translocation, but are not considered here). An example of a fusion protein is a fusion in which an immunoglobulin Fc region is placed at the N-terminus of another protein such as IFN α . Such a fusion is termed an "Fc-X" fusion, where X is a ligand (such as IFN α) and Fc-X proteins have a number of distinctive, advantageous biological properties. In particular, whereas such fusion proteins can still bind the relevant Fc receptors on cell surfaces, when the ligand binds to its receptor, the orientation of the Fc region is altered such that antibody-dependent cell-mediated cytotoxicity and complement fixation are activated by the

30

sequences present in the Fc domain. Fc-X fusions are preferred according to the invention.

The term "immunoglobulin" is used herein to refer to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognised immunoglobulin genes include the κ , λ , α , γ (IgG1, IgG2, IgG3, IgG4), σ , ϵ , and μ constant region genes and in nature multiple immunoglobulin variable region genes.

The term Fc is used herein to refer to an immunoglobulin heavy chain constant region domain and includes the dimeric as well as the monomeric form of the Fc portion of an antibody. Preferably the single chain Fc fusion (monmeric) form is preferred.

The term "T-cell epitope" means according to the understanding of this invention an amino acid sequence which is able to bind MHC class II, able to stimulate T-cells and / or also to bind (without necessarily measurably activating) T-cells in complex with MHC class II.

Reference to "substantially non-immunogenic" or "reduced immunogenic potential" includes reduced immunogenicity compared to a parent protein or to a fusion protein containing the wild-type or native amino acid sequences of the test moiety.

The term "immunogenicity" includes an ability to provoke, induce or otherwise facilitate a humoral and or T-cell mediated response in a host animal and in particular where the "host animal" is a human.

25

The terms "T-cell assay" and "immunogenicity assay" concern *ex vivo* measures of immune reactivity. As such these involve a test immunogen e.g. a protein or peptide being brought into contact with live human immune cells and their reactivity measured. A typical parameter of induced reactivity is proliferation. The presence of suitable control determinations are critical and implicit in the assay.

30

"Time course assay" refers to a biological assay such as a proliferation assay in which determinations of activity are made sequentially over a period of time. In the present context, a "time course T-cell assay", refers to the determination of T-cell proliferation in

response to a test immunogen (peptide) at multiple times following exposure to the test immunogen. The terms "time course T-cell assay" and "time course immunogenicity assay" may be used interchangeably herein.

5 One conventional way in which T-cell assays are expressed is by use of a "stimulation index" or "SI". The stimulation index (SI) is conventionally derived by division of the proliferation score (e.g. counts per minute of radioactivity if using for example ^3H -thymidine incorporation) measured to a test immunogen such as a peptide by the score measured in cells not contacted with a test immunogen. Test immunogens (peptides)
10 which evoke no response give $\text{SI} = 1.0$ although in practice SI values in the range 0.8 - 1.2 are unremarkable. The inventors have established that in the operation of such immunogenicity assays, a stimulation index equal to or greater than 2.0 is a useful measure of significant induced proliferation.

15 PBMC means peripheral blood mononuclear cells in particular as obtained from a sample of blood from a donor. PBMC are readily isolated from whole blood samples using a density gradient centrifugation technique well understood in the art and comprise predominantly lymphocytes (B and T cells) and monocytes. Other cell types are also represented.

20

"Relative activity" means according the present context activity measured for a test protein in any single assay expressed relative to the activity measured for a positive control protein in an identical assay and usually conducted in parallel. Thus if the test protein and the control protein have the same measured activity the relative activity is
25 said to be 1.

An anti-viral assay is a biological assay in which a test protein of interest is measured for any ability to inhibit functioning of a viral agent on suitable host cells. Such an assay is generally configured such that anti-viral activity is becomes equated with prolonged cellular survival or proliferation in the presence of cytopathic doses of virus. For this to
30 be a useful measure suitable control tests are conducted in parallel. The presence of suitable control determinations are critical and implicit in the assay. One particularly suitable antiviral assay is described by Rubinstein et al [Rubinstein S, et al (1981) *J Virol.* 37:755-758] and is exemplified herein. Other assay formats can be contemplated and also

provide quantitative estimations of specific activity of the test molecules to permit ED₅₀ determinations.

A "signalling assay" according to the present context means a biological assay able to
5 provide a reading of the ability of a test protein to evoke a specific measurable response inside a live cell. In particular the test protein is brought into contact with the outside surface of the cell and the measured response is a phenomenon that can occur only with the involvement of a least one specific receptor protein and multiple cellular factors within the cell such as transcription factors. Collectively the receptor and the other
10 multiple cellular factors constitute a "signalling pathway" and such a pathway is known to be activated by functionally active IFN proteins [Williams, B.R. (1991) *Eur. J. Biochem.* 15: 1-11; David, M. (1995) *Pharmacol. Ther.* 65: 149-161]. A particularly suitable signalling assay is exemplified herein, other assay formats can be contemplated to also provide quantitative estimations of specific activity of the test molecules.

15 An "anti-proliferation" assay is a biological assay in which a test protein of interest is measured for any ability to inhibit the growth of an indicator cell culture. For this to be a useful measure suitable control tests are conducted in parallel. One particularly suitable anti-proliferation assay is described by Mark et al [Mark, D.F. (1984) *Proc. Natl. Acad. Sci. USA* 81: 5662-5666] and in modified form is exemplified herein. Other assay
20 formats can be contemplated and also provide quantitative estimations of specific activity of the test molecules to permit ED₅₀ determinations.

In another aspect, the present invention relates to nucleic acids encoding modified IFN α entities.
25 Such nucleic acids are preferably comprised within an expression vector. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilise promoters, enhancers and polyadenylation signals. Such nucleic acids in general comprise a selection means typically an additional gene encoding a protein able to
30 provide for the survival of the host cell. An example of such a selection gene is the beta-lactamase gene suitable for some E.coli host cells and this and others are well known in the art ["Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Gene Transfer Vectors for Mammalian Cells" (J. M. Miller & M. P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F. M. Ausubel et al., eds., 1987)].

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked
5 to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in the same reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at
10 convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

In some embodiments the expression vector comprises a nucleic acid sequence encoding an IFN α variant operably linked to an expression control sequence. In various
15 embodiments the expression vector comprises a nucleic acid sequence encoding a protein selected from the group comprising inclusively SEQ ID No 2 to SEQ ID No 22. Such an expression vector will comprise at least the IFN α encoding domain of one of the said proteins operably linked with suitable expression control and selection sequences. Such an expression vector would include degenerate versions of the nucleic acid wherein
20 degeneracy in relation to polynucleotides refers to the fact well recognised that in the genetic code many amino acids are specified by more than one codon. The degeneracy of the code accounts for 20 different amino acids encoded by 64 possible triplet sequences of the four different bases comprising DNA.

25 Another aspect of the present invention is a cultured cell comprising at least one of the above-mentioned vectors.

A further aspect of the present invention is a method for preparing the modified IFN α comprising culturing the above mentioned cell under conditions permitting expression of
30 the IFN α from the expression vector and purifying the IFN α from the cell.

In a further aspect, the present invention relates to methods for therapeutic treatment of humans using the IFN α compositions. For administration to an individual, any of the

modified compositions would be produced to be preferably at least 80% pure and free of pyrogens and other contaminants. It is further understood that the therapeutic compositions of the IFN α proteins may be used in conjunction with a pharmaceutically acceptable excipient. The pharmaceutical compositions according to the present invention are prepared conventionally, comprising substances that are customarily used in pharmaceuticals, e.g. Remington's Pharmaceutical Sciences, (Alfonso R. Gennaro ed. 18th edition 1990), including excipients, carriers adjuvants and buffers. The compositions can be administered, e.g. parenterally, enterally, intramuscularly, subcutaneously, intravenously or other routes useful to achieve an effect. Conventional excipients include pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral and other routes of administration that do not deleteriously react with the agents. For parenteral application, particularly suitable are injectable sterile solutions, preferably oil or aqueous solutions, as well as suspensions, emulsions or implants, including suppositories. Ampules are convenient unit dosages. The pharmaceutical preparations can be sterilised and, if desired, mixed with stabilisers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers or other substances that do not react deleteriously with the active compounds.

The major embodiments of the present invention are encompassed by the protein sequences SEQ ID Nos 2 – 22. The proteins are fusion proteins of the type "Fc-X" wherein X in this present instance comprise IFN α muteins. Such fusion proteins have been found to show increased activity compared to the fusion proteins containing the wild-type (WT) IFN α moiety. The "WT" or "native" fusion proteins constructed by the inventors herein have been designated IFN120 (SEQ ID No 23) and IFN5 (SEQ ID No 1) and differ only with respect to the presence or absence of a linker peptide of structure (G)₄S(G)₄S(G)₄SG. For clarity, IFN5 does not contain the linker.

Using a signalling assay, the native fusion proteins, either with or without the linker, have been found herein to have very similar ED₅₀ values of 4.5 and 5ng/ml. This finding demonstrates that for native IFN α , the presence of a linker between the Fc and IFN α molecules has no effect on activity.

By contrast, it has been somewhat surprisingly found that the most preferred molecules of the invention IFN311 (SEQ ID No 3) and IFN316 (SEQ ID No 2) have ED₅₀ values of 3.4

- 16 -

and 0.5 ng/ml in a signalling assay and are hence >1.3x and >9x more active than controls. Given that these molecules are IFN α muteins, these results indicate that the changes to the sequence have had a beneficial effect on activity.

- 5 Other IFN α muteins containing two or three or four or five amino acid substitutions were also found with improved relative activity with respect to the signalling assay. Examples each with two substitutions include IFN α proteins IFN197 (SEQ ID No 12), IFN201 (SEQ ID No 11), IFN202 (SEQ ID No 10), IFN306 (SEQ ID No 4) demonstrating respectively 2, 7, 5 and 4 fold improvement in activity. Also proteins IFN173 (SEQ ID
- 10 No 15), IFN176 (SEQ ID No 13), IFN219 (SEQ ID No 9) each with three substitutions demonstrating respectively 10, 5, and 17 fold improvement in activity. IFN α protein IFN174 (SEQ ID No 14) with four substitutions showed a 5 fold improvement in signalling activity.
- 15 Such beneficial effects with respect to activity in a signalling assay are not confined to muteins containing multiple substitutions. Thus for example single mutant IFN α protein IFN28 (SEQ ID No 22) exhibits a 10 fold increased relative activity. Similarly, protein IFN64 (SEQ ID No 21) shows greater than 3 fold increased activity, IFN164 (SEQ ID No 20) shows 3 fold increased activity, proteins IFN167 (SEQ ID No 19) and IFN168 (SEQ
- 20 ID No 18) both show 2 fold improvement, protein IFN171 (SEQ ID No 17) shows a 4 fold improvement and protein IFN172 (SEQ ID No 16) shows a 7 fold improvement with respect to signalling activity.

Whilst signalling activity is a useful indicator of IFN α protein functionality and has been

25 used by the inventors as a rapid screening assay for IFN α muteins, anti-viral activity is the recognised international standard for measuring the potency of IFN α and to a degree a more realistic surrogate of possible clinical activity. Anti-viral activity can therefore be used to compare the activity of the different IFN α molecules and the inventors have used

30 such in the present case to confirm that the most preferred molecules of the invention show activity within the range of clinically validated IFN α preparations. More specifically, fusion protein IFN316 has 13% standard activity, whereas Peg-Intron[®], Pegasys[®] and Albuferon[®] have been reported to have 28%, 10% and 7% respectively [Osborn BL, et al. (2002); *J Pharmacol Exp Ther.* 303:540-548; Grace M, et al (2001) *J*

Interferon Cytokine Res. 21:1103-1115; Bailon P, et al (2001) *Bioconjug Chem.* 12:195-2029,10,11]

The anti-viral activity of the protein is a function of the ability of the protein to evoke the intracellular signalling pathway as extends from the interferon receptor to new gene expression in the nucleus of the cell. Concordance between improvements in signalling activity and anti-viral activity is an expected result and has been shown to be the case for the IFN α muteins of the invention. Thus the IFN α proteins, IFN270 (SEQ ID No 7), IFN273 (SEQ ID No 6) and IFN276 (SEQ ID No 5) each with five substitutions demonstrated respectively a 2 fold, a 3 fold and a further 3 fold improvement in relative signalling activity whilst also demonstrating a greater than 6 fold, a greater than 4 fold and a further greater than 4 fold improvement respectively in relative anti-viral activity. One IFN α mutein showed no improvement in signalling activity being equal to the control in this aspect but yet showed greater than 2 fold (about 2.8) improvement in anti-viral activity. This mutein was protein IFN248 (SEQ ID No 8) and contained 3 substitutions.

The IFN α muteins of the present were constructed to be less immunogenic than the parental molecule. The design of individual muteins was directed from immunological considerations as well as functional activity data. The three regions of immunological importance within the molecule was defined using screening assays involving PBMC preparations from both healthy donor subjects and individuals who had previously received therapeutic IFN α IntronA[®]). Broadly, IFN α muteins were constructed containing mutations within the three identified immunogenic regions. Residues were targeted based upon the known binding properties of HLA-DR molecules in that they have an almost exclusive preference for a hydrophobic amino acid in pocket 1 and that this is the most important determinant of peptide binding [Jardetzky, T.S. et al (1990), *EMBO J.* 9: 1797-1803; Hill, C.M. et al (1994) *J. Immunol.* 152: 2890-2898]. Exhaustive mutational analysis identified those residues within these regions that could be altered without adversely affecting the activity of the fusion protein (Table 2). Choice of alternate residue was guided by the location of the target in the solved NMR structure [Klaus, W. et al (1997), *J. Mol. Biol.* 274: 661-675] and comparison to other human IFN α proteins and those from other species. Buried residues were replaced with either alanine

or similar sized non-hydrophobic residues whereas exposed residues were scanned with all possible non-hydrophobic alternatives.

T-cell assays were also applied in a format to enable fine mapping of the critical residues involved in functional activation of the human T-cells. These studies were done using a family of variant synthetic peptides to scan the region of interest and using known responsive donor samples. Mutation scanning T-cell assays were performed using alanine as the scanning amino acid, except where activity data was already available to guide the choice. Such an approach is able to highlight the contribution of individual amino acid residues to the immunogenicity of the T-cell epitope comprising its locale. Whilst it would be most desired to alter a critical residue involved in the immunogenicity this may not always be compatible with retaining protein function. Multiple substitutions can be employed none of which in isolation are able to eliminate immunogenicity, but which none the less in combination are effective in reducing the immunogenic potential of an otherwise immunogenic region. In the present case, epitope fine mapping studies (Table 4) followed by T-cell assays of combinatorial mutants (Table 5) was able to define combinations of substitutions best able to both retain function and demonstrate reduced immunogenicity in the region of interest.

Further corroborative T-cell assays were conducted using synthetic peptides containing whole combinations of multiple mutation sets (Table 3) to demonstrate reduced immunogenicity in the most desired substitution sets. These latter assays were conducted using synthetic peptides spanning each of the 3 immunogenic regions of the molecule and were run as time-course T-cell assays using PBMCs from both healthy donors and patients who had previously been treated with IntronA[®]. It will be recognised that it is not possible to test purified proteins in T-cell assays due to their anti-proliferative properties.

A general method for the recovery of IFN α muteins with improved properties involves therefore;

- a) identification of T-cell epitopes;
- b) conducting single amino acid substitutions within T-cell epitope regions and selecting functionally active muteins;

- c) optionally, conducting fine mapping studies of critical residues involved in T-cell activation and (optionally) testing double, or triple of more substitutions for immunogenicity;
- d) selecting individual muteins with the most favoured function and immunogenicity profile for constitution as multiply substituted muteins and function testing said same new proteins;
- e) testing functionally active multiply substituted mutein sequences for reduced immunogenicity using time course immunogenicity assays.

10 Taken together, the inventors have been able to define improved IFN α proteins which can be depicted by the following structure:

X⁰-CDLPQTHSLGSRRTLMLLAQMRRX¹SLFSLCKDRHDFGFPQEFGNQFQKA
ETIPVLX²EMX³QQIX⁴NLFSTKDSSAAX⁵DETLLEDKFYTELX⁶QQLNDLEACVI
QGVGVTTETPLMKEDSX⁷LAVRKYFQRITX⁸YLKEKKYSPCAWEVVRAEIMRS

15 FSLSTX⁹LQESLRSKE,

whereby

X⁰ = is Fc or Fc-Linker,

Fc = an Fc domain of an antibody

Linker = a linker peptide consisting of 6 to 25 amino acids

20 X¹ = I, Q

X² = H, Y

X³ = I, T;

X⁴ = F, T, A

X⁵ = W, H;

25 X⁶ = Y, D;

X⁷ = I, N, T, P, R

X⁸ = L, T, H, D, S, N and

X⁹ = N, S;

with the provision that an IFN α molecule wherein simultaneously

30 X¹ = I, X² = H, X³ = I, X⁴ = F, X⁵ = W, X⁶ = Y, X⁷ = I, X⁸ = L and X⁹ = N is excluded.

The following, figures, sequence listing and examples are provided to aid the understanding of the present invention. It is understood that modifications can be made in the procedures set fourth without departing from the spirit of the invention.

DESCRIPTION OF THE SEQUENCES

To aid the understanding of the invention, Table 1 below sets out a description of the fusion protein IFN α muteins. The derivation and properties of these proteins are also more fully disclosed in the examples.

5

Table 1***Description of the sequences***

Clone ID	Substitution(s)*	SEQ ID No
IFN5	None = WT	SEQ ID No 1
IFN316	I24Q, I60T, F64A, W76H, I116R, L128N	SEQ ID No 2
IFN311	I24Q, I60T, F64A, W76H, I116R, L128N	SEQ ID No 3
IFN306	I24Q, W76H	SEQ ID No 4
IFN276	I60T, F64A, W76H, I116T, L128N	SEQ ID No 5
IFN273	I60T, F64A, W76H, I116R, L128N	SEQ ID No 6
IFN270	I60T, F64A, W76H, I116N, L128N	SEQ ID No 7
IFN248	I60T, F64A, W76H	SEQ ID No 8
IFN219	H57Y, F64T, W76H	SEQ ID No 9
IFN202	W76H, I116P	SEQ ID No 10
IFN201	W76H, I116T	SEQ ID No 11
IFN197	W76H, I116N	SEQ ID No 12
IFN176	W76H, L128T, N156S	SEQ ID No 13
IFN174	I60T, W76H, L128T, N156S	SEQ ID No 14
IFN173	I60T, L128T, N156S	SEQ ID No 15
IFN172	L128T	SEQ ID No 16
IFN171	L128S	SEQ ID No 17
IFN168	L128N	SEQ ID No 18
IFN167	L128H	SEQ ID No 19
IFN164	L128D	SEQ ID No 20
IFN64	W76H	SEQ ID No 21
IFN28	Y89D	SEQ ID No 22
IFN120	None = WT	SEQ ID No 23

*The residue numbering for the IFN substitutions commences from residue 1 of the IFN reading frame and is independent of any Fc component.

10

DESCRIPTION OF THE FIGURES

Figure 1 sets out the relative biological activities of each of the preferred IFN α muteins of the invention. The clone ID numbers and the substitutions conducted within each clone are as indicated. The figures denote the relative activities determined for IFN receptor mediated cell activation (= signalling assay), anti-viral activity and anti-proliferation activity using the biological assays set out in Examples. All activities are depicted relative to the Fc-IFN α protein IFN5, which has the WT IFN α moiety in direct fusion to an N-terminal Fc domain.

Figure 2 shows results of receptor signalling assay of cell-culture supernatants. HEK293 cells were transiently transfected with plasmids coding for IFN5, IFN120, IFN316 and IFN311. Protein concentration in the supernatants were quantified by Fc ELISA and diluted to 200ng/ml. The activity was titrated in 3 fold serial dilutions.

Figure 3 shows comparisons of the activity of purified IFN311 and IFN316 to Peprtech IFN α 2a.

- (a) Receptor signalling assay. Titrations were started from 200ng/ml followed by 3 fold serial dilutions.
- (b) Daudi cell anti-proliferation assay. Titrations were started from 200ng/ml followed by 4 fold serial dilutions.
- (c) Anti-viral assay. Initial concentrations were 250pg/ml for IFN311, 62.5pg/ml for IFN316 and 6.25pg/ml for IFN α 2a and titrations were done in 2 fold serial dilutions. Each graph shows data averaged from three experiments.

Figure 4 shows results of time course immunogenicity assays using synthetic peptides (NB: peptide sequences are given in Table 3). 20 healthy individuals and 20 HCV patients (treated with IntronA[®]) were used to assess the immunogenicity of wild type and modified IFN α peptides. Proliferation of PBMCs was assessed by tritiated thymidine incorporation on days 6, 7, 8 and 9 post-stimulation.

- (a) Positive responses (SI>2) from healthy individuals after stimulation with peptides spanning Regions 1, 2 and 3.
- (b) Positive responses (SI>2) from HCV patients after stimulation with peptides spanning Regions 1, 2 and 3.

- (c) Frequency of observed responses with an SI>2 at any time point from a pool of 20 healthy donors and 20 HCV patients to peptides spanning immunogenic Regions 1, 2 and 3.

5 Figures 5.1 to 5.12 show protein sequences of the preferred molecules of the invention. Sequences are depicted in single letter code. The Fc and Fc-linker moiety of each fusion protein is underlined. The IFN α domain is non underlined. The clone ID numbers are shown for each sequence.

10

EXPERIMENTAL EXAMPLES

EXAMPLE 1

Cloning and mutagenesis of IFN α 2b

15 The modified IFN α proteins of the present invention were made using conventional recombinant DNA techniques. The coding sequence for mature IFN α 2b was cloned from human placental DNA (Sigma, Poole, UK) using PCR. The wild-type gene was used both as a control reagent and a template from which to derive modified IFN proteins by site directed mutagenesis. WT and modified genes were inserted into an expression
20 vector pdC-huFc [Lo K-M et al, (1998) *Protein Eng* 11:495-500] such that the IFN α 2b sequence is a direct fusion to the C-terminus of the hinge/CH2/CH3 Fc region of human IgG1. The WT IFN protein in this vector was designated IFN5.

In addition to the direct fusion, a modification of the vector was made which contained a
25 flexible linker between the C-terminus of the CH3 and the N-terminus of IFN α 2b. The amino-acid sequence of this linker was (G)₄S(G)₄S(G)₃SG and the WT IFN protein with the linker was designated IFN120. Some of the mutant IFN proteins were expressed in both vector types thus for example variants IFN311 (SEQ ID No 3) and IFN316 (SEQ ID No 2) whilst comprising identical substitution sets within their IFN domains differ in
30 respect of the (G)₄S(G)₄S(G)₃SG linker. IFN311 is a direct fusion, whereas IFN316 contains the linker.

DNA sequencing was conducted on all constructs. This was diligently performed to confirm introduction of desired substitutions and establish that no extraneous (undesired) substitutions had been introduced for example by PCR error.

Details of the techniques and cloning strategy for the WT and variant IFN proteins have been detailed elsewhere [WO 02/085941] and are commonly understood in the art.

EXAMPLE 2

5 Design of IFN α muteins

Variants of IFN α 2b linked to the Fc portion of human IgG1 were constructed containing mutations within the three immunogenic regions of the protein. Cycles of mutational analysis involving construction and function testing identified those residues within these regions that could be altered without adversely affecting the activity of the Fc-linked protein. The signalling assay as described herein (see Example 5) was the main screening tool in this aspect. Results of the mutational analysis are shown in Table 2.

Table 2

IFN α amino acid residues targeted for mutation

- 15 * indicates that all non-hydrophobic alternatives were tested with most active shown
+determined by receptor signalling assay and compared to Fc linked wt IFN α .

	Region 1				
	I ²⁴	L ²⁶	F ²⁷	L ³⁰	F ³⁶
Substitution	P/Q*	H*	S	A	A
Relative Activity ⁺	4	0.25	0.1	0	0

	Region 2					
	I ⁶⁰	I ⁶³	F ⁶⁴	L ⁶⁶	F ⁶⁷	W ⁷⁶
Substitution	T	T	A*	T*	A	H
Relative Activity ⁺	1	0.5	0.8	0	0	5

20

	Region 3						
	I ¹¹⁶	L ¹¹⁷	V ¹¹⁹	Y ¹²²	F ¹²³	I ¹²⁶	L ¹²⁸
Substitution	T*	H*	A	H	H	A/T	T*
Relative Activity ⁺	2	0.9	0.5	0	0.1	0.1	7

Choice of alternate residue was guided by the location of the target in the solved NMR structure [Klaus, W. et al (1997), *J. Mol. Biol.* 274: 661-675] and comparison to other human IFN α proteins and those from other species. Buried residues were replaced with either alanine or similar sized non-hydrophobic residues whereas exposed residues were
5 scanned with all possible non-hydrophobic alternatives.

In Region 1, the only hydrophobic residue that could be replaced successfully, with a significant improvement in activity, was I24. L26, F27 and L30, although well exposed, could not be changed presumably because they form a well-defined hydrophobic patch
10 with inter-molecular functionality.

In Region 2, the only well-exposed residue was F64 which was changed to A with a modest drop in activity. Residues I60 and I63, although mostly buried, were successfully changed to T, a residue of similar size that can add hydrogen bonds to the alpha-helix in
15 which these residues lie. Residue W76, which lies towards one tip of the molecule, although substantially buried, was changed to H with a significant benefit in activity.

In Region 3, the only residues that could be successfully altered were those that were well exposed. Changes to I116 and L128 gave significant benefits in activity and alteration of
20 L117 resulted in slightly reduced activity.

Data from scanning T-cell assays conducted on immunogenic Region 3 were also included to further refine the design process (see Tables 4 and 5, Example 9). Whereas immunologically the most favoured single substitution would have been F123, this was
25 not compatible with any useful level of signalling activity. By contrast, the combination of substitutions at residues I116 and L128 were confirmed to modulate the T-cell response to this region. Further substitutions at each position were tested to encompass a range of sizes of side chains that had shown good activity. The substitutions were I116 to T, N and R and L128 to N, H and R. Nine mutants were made containing each
30 combination of these residues in conjunction with those mutations in Regions 1 and 2 that gave acceptable activity (I24Q, I60T, F64A, W76H).

A combined mutein containing I116R and L128N together with I24Q, I60T, F64A, and W76H was constructed in a format that either contained or did not contain a flexible linker between the Fc and IFN α fusion partners (IFN316 and IFN311 respectively).

5 EXAMPLE 3.

Transfection and purification of fusion proteins

Transient transfections were done using HEK293 (ATCC# CRL-1573) cells and Lipofectamine 2000 (Invitrogen, Paisley, UK) as described by the manufacturer. Stable transfectants were made in NS0 cells (ECACC# 85110503) using electroporation as
10 previously described [Baum C, et al (1994) *Biotechniques* 17:1058-106224] and selected in media containing 100nM methotrexate. All cell-lines were maintained in DMEM plus 10% FBS with antibiotics and antimycotics. Fusion proteins were purified via Prosep-A chromatography followed by size exclusion chromatography (SEC). Briefly, 1ml Prosep[®]-A columns (Millipore, Watford, UK) were equilibrated in PBS pH 7.4 before
15 being loaded with 0.2 μ M filtered cell-culture supernatants (up to 500ml) that had been pH adjusted with 1/20 vols. 1M Tris-HCl pH 7.4. The column was washed with 50ml PBS pH 7.4 and the fusion protein eluted with 0.1M citrate buffer pH 3.0 and 0.9ml fractions collected. The fractions were immediately neutralized with 0.1ml 1M Tris-HCl pH 8.0. SEC was done with Superdex 200 (Amersham Pharmacia, Amersham, UK) in a 3.2/30
20 column equilibrated and run in PBS pH 7.4 containing 0.1% Tween 80. Fractions spanning the major peak were pooled and fusion proteins quantified using molar extinction coefficients at 280nm calculated using Lasergene[™] software (Dnastar, Madison, WI, USA). The concentrations were confirmed using a BCA protein assay (Pierce, Chester, UK). The IFN α component represents 42% of the molecular weight of
25 the fusion protein, therefore the concentrations were adjusted by this factor.

EXAMPLE 4

Quantitation of fusion proteins in cell-culture supernatants

Fusion proteins were quantified by detecting the amount of human IgG1 Fc in an ELISA
30 format as follows: ELISA plates (Dynex Immulon4) were coated with a mouse monoclonal anti-human IgG Fc specific antibody at a dilution of 1/1500 in PBS pH7.4, 100 μ l/well, for 2h at 37°C. The plate was washed x4 with 100 μ l/well PBS/0.05% Tween 20. Human IgG standards (The Binding Site, Birmingham, UK) were diluted to 2 μ g/ml

in PBS/2%BSA and duplicate two-fold dilutions made vertically down the plate. Test samples were diluted 1/100 and 1/500 in PBS/2% BSA and assayed in duplicate. The plate was incubated for 1h at room temperature and washed as before. Detection was done using 100µl/well goat anti-human IgG Fc-specific peroxidase conjugate (The Binding Site, Birmingham, UK) at a dilution of 1/1000 in PBS, the plate washed as before and colour developed using SigmaFast OPD, 100µl/well (Sigma, Poole, UK). The colour reaction was stopped by the addition of 50µl 2M sulphuric acid and the absorbance measured at 492nm in an Anthos HTII plate reader.

10 EXAMPLE 5

Assays for Fc-IFN α 2b activity (signalling assay)

Plasmids coding for variant and WT IFN fusion proteins were transfected transiently into HEK293 cells and after three days the cell-culture supernatants were quantified for the Fc portion of the fusion. The supernatants were assayed for activity using a signalling assay.

15 Measurement of activity in this assay requires triggering (activation) of the type I interferon receptor expressed on the cell surface. The activated receptor leads to the activation of the Jak/STAT1 signalling pathway within the cell. The pathway culminates in phosphorylation of protein STAT1 enabling it to bind the Interferon Stimulated Response Element (ISRE). The ISRE is a cis-acting DNA segment which is able to

20 promote transcription of genes (e.g. a reporter gene) linked downstream to it.

The ability of the fusion proteins of the invention to induce signalling from the type I interferon receptor was assayed using a commercially supplied signalling reporter vector, pISRE-TA-luc (Clontech Europe, Brussels, Belgium). The vector contains the firefly

25 luciferase gene under the control of the Interferon Stimulated Response Element (ISRE). The ISRE/luciferase cassette (on a NotI/BamHI fragment) was transferred to the episomal mammalian expression vector pREP4 (Invitrogen, Paisley, UK) in place of the RSV promoter/MCS/SV40 polyA (removed via SalI digestion), to create pREP-ISRE. This vector was transfected into HEK293 cells and stable transfectants selected with 100µg/ml

30 hygromycin to create HEK-ISRE cells. Assays for Fc-IFN α 2b activity were done by plating HEK-ISRE cells at a density of 4×10^5 cells/ml into the wells (100µl/well) of black walled, clear bottomed 96 well luminometer plates (Greiner, Stonehouse, Glouc., UK) and incubating for 24h under normal conditions in the absence of antibiotics.

Duplicate serial dilutions of standard IFN α 2a 2×10^8 IU/mg (Peprtech, London, UK) and fusion proteins in antibiotic free media were made down the plate and incubated overnight. Luciferase activity was detected by the addition of 100 μ l Steady-Glo reagent (Promega, Southampton, UK) prepared as instructed by the supplier, followed by
5 measurement of luminescence using a Wallac Microbeta Trilux luminometer.

Figure 1 tabulates the relative activities of each IFN α mutein. Figure 2 shows signalling activity curves for fusion proteins IFN5, IFN120, IFN311 and IFN316. These results show that the variant fusion proteins have increased activity compared to the native fusion
10 proteins (IFN120 and IFN5, with and without the linker respectively). The native fusion proteins, either with or without the linker, have very similar ED₅₀ values of 4.5 and 5ng/ml respectively, demonstrating that, for native IFN α , the presence of a linker between the Fc and IFN α molecules has no effect on activity. IFN311 and IFN316 have ED₅₀ values of 3.4 and 0.5 ng/ml and are hence >1.3x and >9x more active than controls,
15 demonstrating that the changes to the sequence have had a beneficial effect on activity. A surprising result in this assay was found in that in contrast to the native IFN α constructs, the presence of a linker between the Fc and IFN α increased the activity of the fusion protein.

Signalling activity of fusion proteins IFN311 and IFN316 were also compared to a native
20 IFN α preparation (Peprtech, London, UK) and the activity curves are depicted in Fig. 3a.

EXAMPLE 6

Activities of the modified Fc-IFN α 2b fusion proteins (anti proliferation):

25 The anti-proliferative properties of the fusion proteins were assessed by inhibition of Daudi cell proliferation and were performed as follows: Daudi cells (ATCC# CCL-213) were grown in RPMI1640 plus 10% FBS plus antibiotics (Daudi media). Cells in mid log phase were diluted to 2×10^5 cells/ml and plated out at 75 μ l/well into 96 well microtitre plates. Dilutions of standard IFN α 2a (Peprtech) and fusion proteins were made in
30 triplicate in 75 μ l Daudi media and added to the cells. Serial $\frac{1}{4}$ dilutions were made across the plate; therefore 1 standard and one test sample were assayed per plate. The plates were incubated for 3 days. Detection was done using Aqueous One reagent (Promega) prepared as described by the manufacturer. 30 μ l reagent was added to each

well, the plates incubated for 4h and the absorbance at 492nm read using an Anthos HTII plate reader.

Figure 3b shows the anti-proliferative properties plotted for fusion proteins IFN311 and IFN316 relative to a native (non fusion protein) $\text{INF}\alpha$ preparation (Peprotech).

5

EXAMPLE 7

Activities of the modified Fc- $\text{INF}\alpha 2b$ fusion proteins (anti-viral):

The antiviral properties of the fusion proteins were assayed by the inhibition of cytopathic effect assay of encephalomyocarditis virus (EMCV) on human lung carcinoma A549 cells
10 (ATCC# CCI-185) as previously described [Rubinstein S, et al (1981) *J Virol.* 37: 755-758].

Figure 1 tabulates the relative activities of several $\text{INF}\alpha$ muteins in this assay. The maximum relative activity was shown by fusion protein IFN316, where the calculated
15 fold increase over the control was 36. The surprising result is that the presence of the linker in IFN316 has a marked beneficial effect on its anti-viral activity compared to the linker free counterpart IFN311 (relative activity = 7.4). This marked difference is not seen in comparing the WT $\text{INF}\alpha$ fusion proteins with and without the linker (IFN5 versus IFN120). Figure 3c shows the anti-viral activity properties plotted for fusion proteins
20 IFN311 and IFN316 relative to a native (non fusion protein) $\text{INF}\alpha$ preparation (Peprotech).

EXAMPLE 8

T-cell epitope mapping of human $\text{INF}\alpha$ and analysis of immunogenic regions by 25 time-course T-cell assays:

The initial T-cell epitope mapping study of human $\text{INF}\alpha$ was conducted using synthetic peptides and PBMC from healthy donors and the results and details of this assay have been described elsewhere [WO 02/085941]. Further analysis of the three immunogenic regions identified within $\text{INF}\alpha$ and termed R1, R2 and R3, was conducted using a time-
30 course T-cell assay. This assay was performed using PBMCs isolated from blood donated by 20 healthy individuals (selected to cover >80% of common HLA-DR alleles) and also from 20 patients with chronic HCV infection previously treated with $\text{INF}\alpha 2b$ (IntronA[®]) according to NICE guidelines (patient studies were conducted under

collaboration with Dr G. Alexander, Addenbrooke's Hospital, Cambridge, UK). Immunogenic epitopes R1, R2 and R3 were tested as synthetic peptides as the immunoactive properties of the intact IFN α proteins are not compatible with this assay. In these assays, bulk cultures of $2-4 \times 10^6$ PBMC/well of a 24 well plate were incubated for 6 to 9 days with peptides spanning the immunogenic regions (see Table 3). Proliferation was assessed at various time points by gently resuspending the bulk cultures and removing samples of PBMC that were then incubated in triplicate wells of U-bottomed 96 well plate with 1 μ Ci/well tritiated thymidine for 18 hours before harvesting onto glass fibre filter mats using a Tomtec Mach III plate harvester and cpm values determined by scintillation counting using a Wallac Microplate Beta counter.

Table 3.***Sequences of Peptides Used in Time-Course Assays***

Epitope region	Wild type sequence
R1	QMRRISLFSLKDRHDFGF
R2	EMIQQIFNLFSTKDSSAAWDETLLDKFY
R3	TPLMKEDSILAVRKYFQRITLYLKEKKYSPCAW

Epitope region	Modified sequence*
R1	QMRRQSLFSLKDRHDFGFP
R2	EMTQQIANLFSTKDSSAAHDETLLDKFY
R3	TPLMKEDSRLAVRKYFQRITNYLKEKKYSPCAW

*Amino acid substitutions constituting the modified sequences are shown underlined.

15

Figure 4a shows all positive responses (SI>2) by healthy individuals to stimulation with peptides spanning immunogenic regions 1, 2 and 3. Modified peptides failed to induce proliferation in any healthy individuals whereas positive responses to wild type peptides were observed in six donors (Figure 4a). HCV patients were also found to respond to wild type peptides of all three regions whereas for the modified peptides they only responded to Region 3 (Figure 4b). Analysis of the frequency of responses to peptides showed that Regions 2 and 3 appeared to induce the most frequent proliferation, in 15% of the healthy donors tested (Figure 4c), whereas in HCV patients Region 3 induced the highest frequency of responses (25%) followed by Region 1 (10%) and then Region 2 (5%) (Figure 4c).

25

From this and other data, Region 3 is considered to contain an immunodominant T-cell epitope since the results from the T-cell epitope mapping show that peptides derived from Region 3 also induce proliferation with the highest frequency in healthy donors [WO 02/085941]. For healthy individuals, responses to the Region 1 peptide were observed on day 7 (Figure 4a, donor 11 and donor 13), whereas proliferation was detected on day 6 in the only HCV patient T-cell response. The Region 2 peptide tended to induce proliferation on day 9 in healthy donors (Figure 4a, donor 6 and donor 18) whereas the only HCV patient T-cell response was observed on day 8 (Figure 4c, donor 13). Unlike Region 1 and 2 responses, the Region 3 peptide induced a more rapid response in healthy individuals than in HCV patients with proliferation observed on days 7 and 8, respectively.

EXAMPLE 9

Scanning T cell assays of IFN α immunogenic region 3

Data from scanning T-cell assays conducted on immunogenic Region 3 were also included to further refine the mutein design process. T-cell assays were conducted using PBMC from 13 donor samples the majority of which were pre-determined to be responsive to at least the WT peptide sequence of interest. A family of 8 synthetic peptides were produced spanning residues T108-W140 of IFN α . The family of peptides contained the WT sequence and 7 different substituted sequences as identified in Table 4. All assays were conducted in triplicate. The mean SI across the 13 donor samples was determined.

Table 4

Mutation Scanning T-cell assays of IFN α immunogenic region 3

	I ¹¹⁶	L ¹¹⁷	V ¹¹⁹	Y ¹²²	F ¹²³	I ¹²⁶	L ¹²⁸
Substitution	S	A	A	H	H	A	A
Mean SI*	1.58	1.53	2.46	-	1.02	1.09	1.82
Frequency SI>1.95	15%	15%	15%	-	0%	15%	23%

*T-cell assays were done with peptides spanning IFN α residues T108-W140. Wild type peptide produced an average SI of 2.12 with a 15% frequency of responses.

- 5 Alanine was used as the scanning amino acid, except where activity data was already available to guide the choice (Y122 was not scanned since mutants were not active). F123H was found to be the most effective single mutation in reducing the overall T cell response with the SI being consistently less than wild-type over a panel of 13 donors, however F123 could not be mutated to recover sufficient activity. Several of the changes
10 gave an equivalent frequency of positive response to WT (Table 4) but they did not respond to the same subset of donors, although there was some overlap. The mean SI of the mutants were generally lower than WT peptide, even for L128A which gave an increased frequency of positive responses, although V119A gave consistently higher SI over the donor set but the frequency of positive responses was similar to WT.
- 15 Alternative combinations of region 3 muteins were tested using immunogenicity assay. Nine peptides were synthesised spanning Region 3, containing each combination of amino acids, and re-tested in the T-cell proliferation assay (Table 5).

Table 5

20 ***Immunogenicity of Region 3 muteins***

Region 3 Mutation	Mean SI [#] (frequency SI>1.95)
WT	1.43 (15%)
I116R + L128N	1.13
I116R + L128H	1.21
I116R + L128R	1.17
I116N + L128N	1.15
I116N + L128H	1.14
I116N + L128R	1.21 (5%)
I116T + L128N	1.10
I116T + L128H	1.08
I116T + L128R	1.07

- 32 -

#T-cell assays were done with peptides spanning IFN α residues T108-W140, containing the Region 3 mutations indicated in the left hand column

15% of donors responded to the WT peptide with an SI > 1.95. The mean SI for the WT
5 peptide over all donor samples was 1.43. All combinations of changes gave mean SI over a panel of 20 donors that were less than WT peptide, however the peptide containing I116N + L128R did give a positive response (SI>1.95) in one donor.

PATENT CLAIMS

1. A modified human interferon alpha 2 (IFN α 2) molecule having improved biological and immunogenic properties having the amino acid sequence:

5 X⁰-CDLPQTHSLGSRRTLMLLAQMRRX¹SLFSLCKDRHDFGFPQEEFGNQFQKA
 ETIPVLX²EMX³QQIX⁴NLFSTKDSSAAX⁵DETLDDKIFYTELX⁶QQLNDLEACVI
 QGVGVTTETPLMKEDSX⁷LAVRKYFQRITX⁸YLKEKKYSPCAWEVVRAEIMRS
 FSLSTX⁹LQESLRSKE,

whereby

10 X⁰ = is Fc or Fc-Linker,
 Fc = an Fc domain of an antibody
 Linker = a linker peptide consisting of 6 to 25 amino acids

X¹ = I, Q
 X² = H, Y
 15 X³ = I, T;
 X⁴ = F, T, A
 X⁵ = W, H;
 X⁶ = Y, D;
 X⁷ = I, N, T, P, R
 20 X⁸ = L, T, H, D, S, N and
 X⁹ = N, S;

with the provision that an IFN α molecule wherein simultaneously
 X¹ = I, X² = H, X³ = I, X⁴ = F, X⁵ = W, X⁶ = Y, X⁷ = I, X⁸ = L and X⁹ = N
 is excluded.

25
2. An IFN α 2 mutein according to claim 1, wherein
 X⁵ = H and X⁸ = N.
3. An IFN α 2 mutein according to claim 2, wherein
 30 X³ = T and X⁴ = A.
4. An IFN α 2 mutein of any of the claims 1 to 3, selecting from the group consisting of the following compounds:

(i) X¹ = Q, X² = H, X³ = T, X⁴ = A, X⁵ = H, X⁶ = Y, X⁷ = R, X⁸ = N and X⁹ = N

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- (ii) $X^1 = Q, X^2 = H, X^3 = I, X^4 = F, X^5 = H, X^6 = Y, X^7 = I, X^8 = L$ and $X^9 = N$
 (iii) $X^1 = I, X^2 = H, X^3 = T, X^4 = A, X^5 = H, X^6 = Y, X^7 = T, R$ or $N, X^8 = N$ and $X^9 = N$
 (iv) $X^1 = I, X^2 = H, X^3 = T, X^4 = A, X^5 = H, X^6 = Y, X^7 = I, X^8 = L$ and $X^9 = N$
 (v) $X^1 = I, X^2 = Y, X^3 = I, X^4 = T, X^5 = H, X^6 = Y, X^7 = I, X^8 = L$ and $X^9 = N$
 5 (vi) $X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = H, X^6 = Y, X^7 = P, T$ or $N, X^8 = L$ and $X^9 = N$
 (vii) $X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = H, X^6 = Y, X^7 = I, X^8 = T$ and $X^9 = S$
 (viii) $X^1 = I, X^2 = H, X^3 = T, X^4 = F, X^5 = H, X^6 = Y, X^7 = I, X^8 = T$ and $X^9 = S$
 (ix) $X^1 = I, X^2 = H, X^3 = T, X^4 = F, X^5 = W, X^6 = Y, X^7 = I, X^8 = T$ and $X^9 = S$
 (x) $X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = W, X^6 = Y, X^7 = I, X^8 = T, S, N, H$ or D and
 10 $X^9 = N$
 (xi) $X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = H, X^6 = Y, X^7 = I, X^8 = L$ and $X^9 = N$
 (xii) $X^1 = I, X^2 = H, X^3 = I, X^4 = F, X^5 = W, X^6 = D, X^7 = I, X^8 = L$ and $X^9 = N$

5. An IFN α 2 mutein according to any of the claims 1 to 4, wherein Fc is a human
 15 immunoglobulin heavy chain constant region domain, which is linked by its C-terminus to said mutein.
6. An IFN α 2 mutein according to claim 5, wherein the Fc domain is a monomer.
- 20 7. An IFN α 2 mutein according to any of the claims 1 – 6, wherein the linker peptide consists of 12 to 20 amino acids.
8. An IFN α 2 mutein according to claim 7, wherein the linker peptide is
 (G)4S(G4)S(G4) SG
- 25 9. An IFN α 2 mutein according to any of the claims 1 – 8, wherein the enhanced immunogenic properties result in a loss of immunogenicity, whereby said loss of immunogenicity is achieved by deleting MHC Class II-binding T-cell epitopes presented on the originally non-modified molecule.
- 30 10. An IFN α 2 mutein according to claim 9 exhibiting when tested as a whole protein in a biological T-cell proliferation assay a stimulation index (SI) smaller than the parental non-modified IFN molecule and smaller than 2.

11. A DNA sequence coding for an IFN α 2 mutein of any of the claims 1 – 10.
12. A pharmaceutical composition comprising an IFN α 2 mutein as defined in any of the
above-cited claims, optionally together with a pharmaceutically acceptable carrier,
5 diluent or excipient.
13. Use of an IFN α 2 mutein of any of the claims 1 – 11 for the manufacture of a
medicament for the treatment of a patient suffering from hepatitis C virus infection.

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FIGURE 2

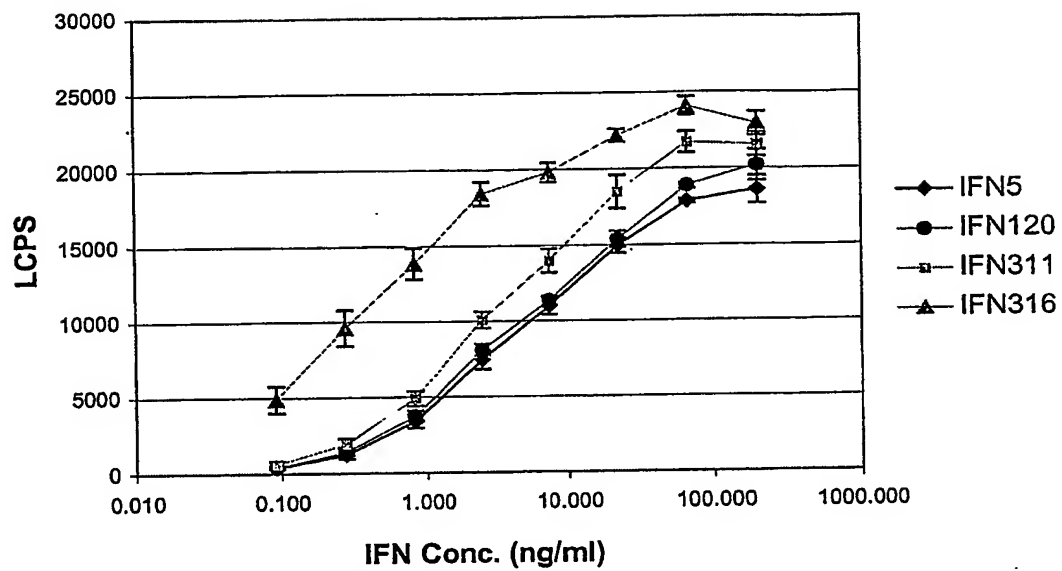


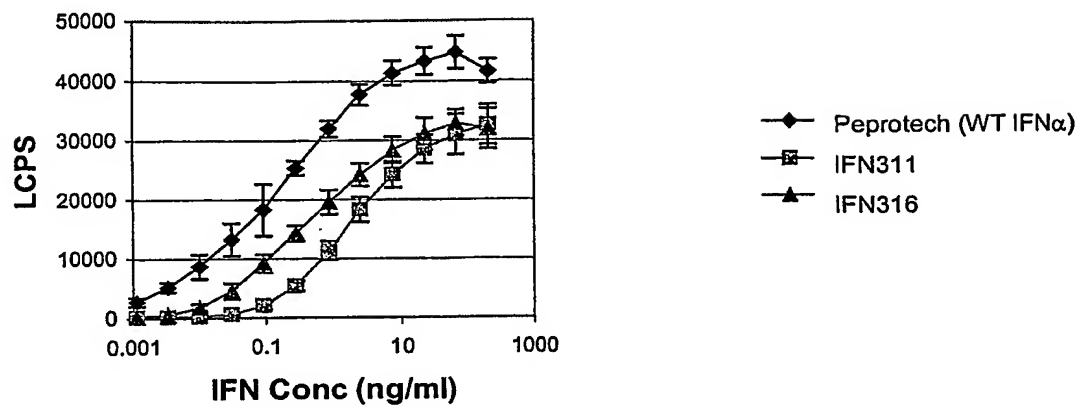
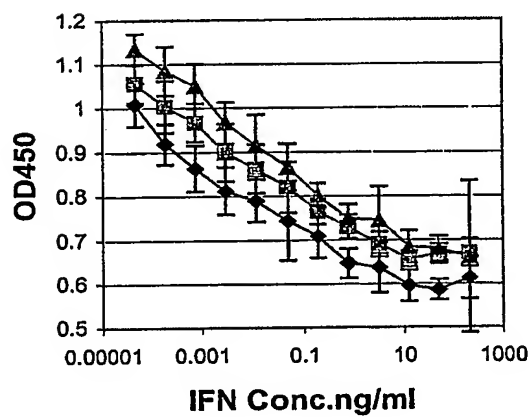
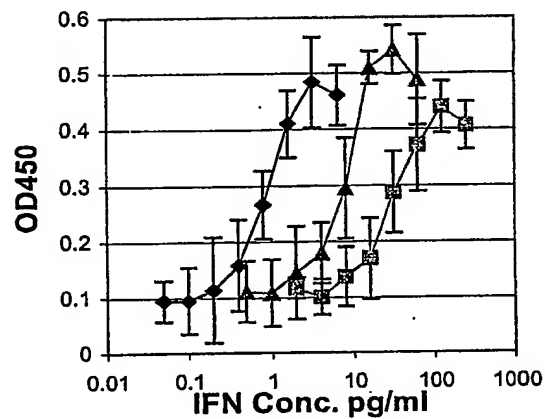
FIGURE 3**(a)****(b)****(c)**

FIGURE 4a

(a)

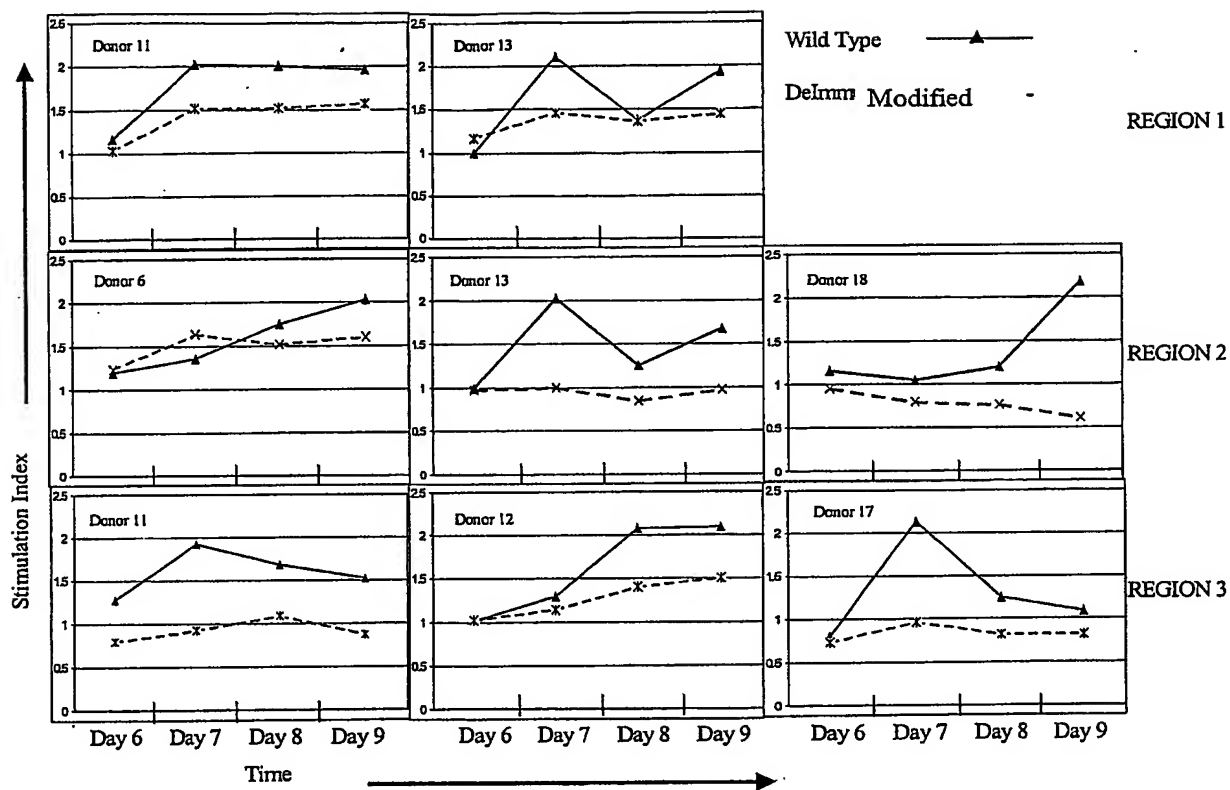
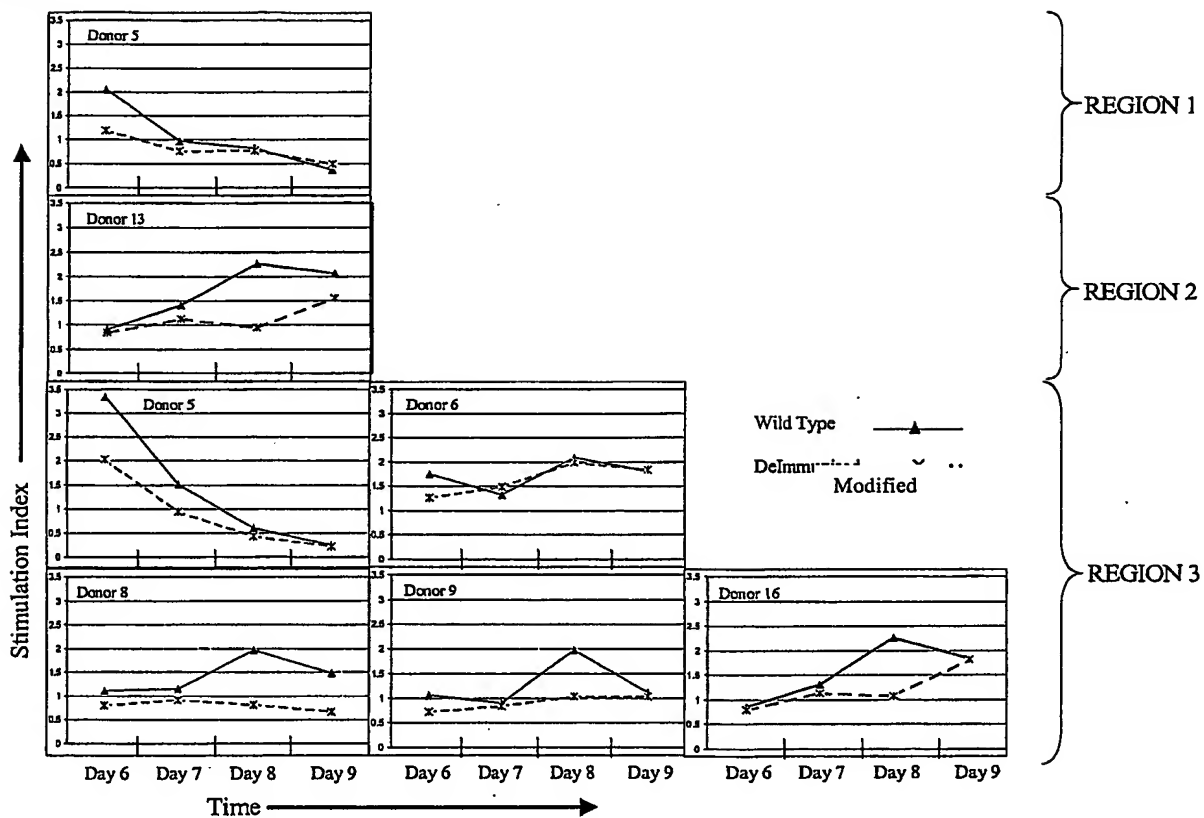


FIGURE 4b

(b)



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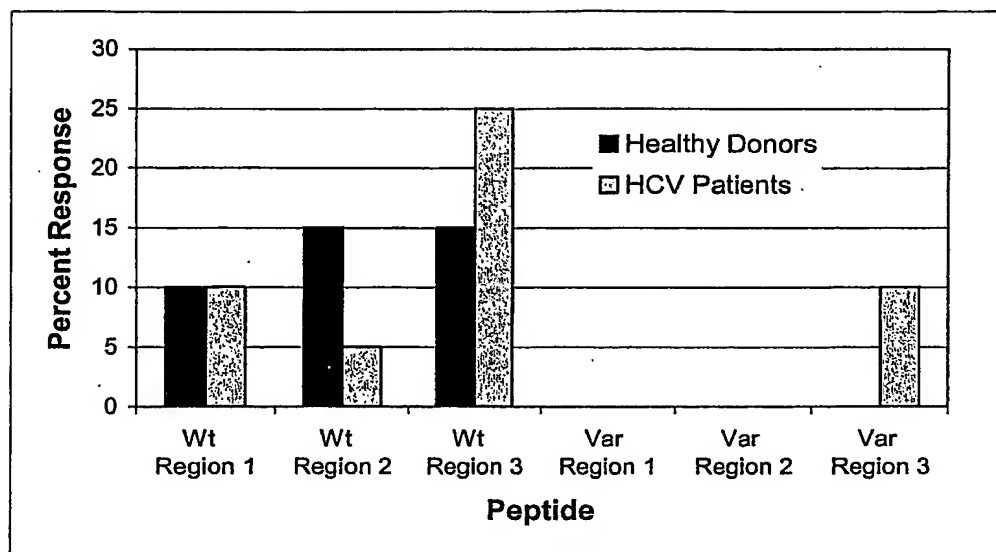
FIGURE 4c**(c)**

FIGURE 5.1

IFN5:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTILMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHODWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	POVYTLPPSR	EEMTKNOVSL
160	170	180	190	200
TCLVKGFPYS	DIAVEWESNG	QPENNYKTTP	PVLDSGSGFF	YSKLTVDKSR
210	220	230	240	250
WQQGNVFSCS	VMHEALHNHY	TQKSLSLSPG	KCDLPQTHSL	GSRRTLMILA
260	270	280	290	300
QMRRISLFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	IQQIFNLFST
310	320	330	340	350
KDSSAAWDET	LIDKFYTELY	QQLNDLEACV	IQGVGVTTETP	LMKEDSILAV
360	370	380	390	
RKYFORITLY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNIQE	SLRSKE

IFN28:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTILMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHODWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	POVYTLPPSR	EEMTKNOVSL
160	170	180	190	200
TCLVKGFPYS	DIAVEWESNG	QPENNYKTTP	PVLDSGSGFF	YSKLTVDKSR
210	220	230	240	250
WQQGNVFSCS	VMHEALHNHY	TQKSLSLSPG	KCDLPQTHSL	GSRRTLMILA
260	270	280	290	300
QMRRISLFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	IQQIFNLFST
310	320	330	340	350
KDSSAAWDET	LIDKFYTELD	QQLNDLEACV	IQGVGVTTETP	LMKEDSILAV
360	370	380	390	
RKYFORITLY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNIQE	SLRSKE

FIGURE 5.2

IFN164:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTILMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	POVYTLPPSR	EEMTKNQVSL
160	170	180	190	200
TCLVKGFPYS	DIAVEWESNG	OPENNYKITP	PVLDSGSGFF	YSKLTVDKSR
210	220	230	240	250
WQOQNVFSCS	VMHEALHNHY	TQKSLSLSPG	KCDLPQTHSL	GSRRITMLLA
260	270	280	290	300
QMRRISLFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	IQQIFNLFS
310	320	330	340	350
KDSSAAHDET	LDDKFTYELY	QQLNDLEACV	IQGVGVTTTP	LMKEDSILAV
360	370	380	390	
RKYFORITLY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE

IFN164:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTILMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	POVYTLPPSR	EEMTKNQVSL
160	170	180	190	200
TCLVKGFPYS	DIAVEWESNG	OPENNYKITP	PVLDSGSGFF	YSKLTVDKSR
210	220	230	240	250
WQOQNVFSCS	VMHEALHNHY	TQKSLSLSPG	KCDLPQTHSL	GSRRITMLLA
260	270	280	290	300
QMRRISLFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	IQQIFNLFS
310	320	330	340	350
KDSSAAWDET	LDDKFTYELY	QQLNDLEACV	IQGVGVTTTP	LMKEDSILAV
360	370	380	390	
RKYFORITDY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE

FIGURE 5.3

IFN167:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDDEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL
160	170	180	190	200
TCLVKGFPYS	DIAVEWESNG	OPENNYKTTT	PVLDSGSGFF	YSKLTVDKSR
210	220	230	240	250
WQQGNVFSCS	VMHEALHNHY	TQKSLSLSPG	KCDLPQTHSL	GSRRITMLLA
260	270	280	290	300
QMRRISLFSC	LKDRHDFGPP	QEEFGNQFQK	AETIPVLHEM	IQQIFNLFST
310	320	330	340	350
KDSSAAWDET	LDDKFYTELY	QQLNDLEACV	IQGVGVTEPT	LMKEDSILAV
360	370	380	390	
RKYFORITHY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE

IFN168:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDDEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL
160	170	180	190	200
TCLVKGFPYS	DIAVEWESNG	OPENNYKTTT	PVLDSGSGFF	YSKLTVDKSR
210	220	230	240	250
WQQGNVFSCS	VMHEALHNHY	TQKSLSLSPG	KCDLPQTHSL	GSRRITMLLA
260	270	280	290	300
QMRRISLFSC	LKDRHDFGPP	QEEFGNQFQK	AETIPVLHEM	IQQIFNLFST
310	320	330	340	350
KDSSAAWDET	LDDKFYTELY	QQLNDLEACV	IQGVGVTEPT	LMKEDSILAV
360	370	380	390	
RKYFORITNY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE

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FIGURE 5.4

IFN171:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	BEWTKNQVSL
160	170	180	190	200
TCLVKGFYPS	DIAVEWESNG	OPENNYKTTP	PVLDSGDSFF	YSKLTVDKSR
210	220	230	240	250
WQOQNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTMLLLA
260	270	280	290	300
QMRRISLFSC	LKDRHDFGPP	QEEFGNQFOK	AETIPVLHEM	IQQIFNLFST
310	320	330	340	350
KDSSAAWDET	LLDKFYTELY	QQLNDEACV	IQGVGVTETP	LMKEDSILAV
360	370	380	390	
RKYFORITSY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE

IFN172:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	BEWTKNQVSL
160	170	180	190	200
TCLVKGFYPS	DIAVEWESNG	OPENNYKTTP	PVLDSGDSFF	YSKLTVDKSR
210	220	230	240	250
WQOQNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTMLLLA
260	270	280	290	300
QMRRISLFSC	LKDRHDFGPP	QEEFGNQFOK	AETIPVLHEM	IQQIFNLFST
310	320	330	340	350
KDSSAAWDET	LLDKFYTELY	QQLNDEACV	IQGVGVTETP	LMKEDSILAV
360	370	380	390	
RKYFORITTY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE

FIGURE 5.5

<u>IFN173:</u>					
10	20	30	40	50	
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD	
60	70	80	90	100	
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN	
110	120	130	140	150	
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	POVYTLPPSR	EEMTKNQVSL	
160	170	180	190	200	
TCLVKGFYPS	DIAVEWESNG	OPENNYKTTP	PVLDSDGSFF	YSKLTVDKSR	
210	220	230	240	250	
WQOQNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTMLLA	
260	270	280	290	300	
QMRRISLWSC	LKDRHDFGFP	QEEFGNQFQK	AETIPVLHEM	TQQIFNLFST	
310	320	330	340	350	
KDSSAAWDET	LLDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSILAV	
360	370	380	390		
RKYFORITTY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTSLOE	SLRSKE	

<u>IFN174:</u>					
10	20	30	40	50	
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD	
60	70	80	90	100	
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN	
110	120	130	140	150	
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	POVYTLPPSR	EEMTKNQVSL	
160	170	180	190	200	
TCLVKGFYPS	DIAVEWESNG	OPENNYKTTP	PVLDSDGSFF	YSKLTVDKSR	
210	220	230	240	250	
WQOQNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTMLLA	
260	270	280	290	300	
QMRRISLWSC	LKDRHDFGFP	QEEFGNQFQK	AETIPVLHEM	TQQIFNLFST	
310	320	330	340	350	
KDSSAAWDET	LLDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSILAV	
360	370	380	390		
RKYFORITTY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTSLOE	SLRSKE	

FIGURE 5.6

IFN176:					
10	20	30	40	50	
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD	
60	70	80	90	100	
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN	
110	120	130	140	150	
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL	
160	170	180	190	200	
TCLVKGFPYS	DIAVEWESNG	QPENNYKTTP	PVLDSGSGFF	YSKLTVDKSR	
210	220	230	240	250	
WQQGNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTLMLLA	
260	270	280	290	300	
QMRRI SLFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	IQQIFNLFST	
310	320	330	340	350	
KDSSAAHDET	LLDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSNLAV	
360	370	380	390		
RKYFQRITTY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTSLQE	SLRSKE	

IFN197:					
10	20	30	40	50	
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD	
60	70	80	90	100	
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN	
110	120	130	140	150	
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL	
160	170	180	190	200	
TCLVKGFPYS	DIAVEWESNG	QPENNYKTTP	PVLDSGSGFF	YSKLTVDKSR	
210	220	230	240	250	
WQQGNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTLMLLA	
260	270	280	290	300	
QMRRI SLFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	IQQIFNLFST	
310	320	330	340	350	
KDSSAAHDET	LLDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSNLAV	
360	370	380	390		
RKYFQRITLY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTSLQE	SLRSKE	

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FIGURE 5.7

IFN201:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYCKKVS	NALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL
160	170	180	190	200
TCLVKGFP	YS DIAVEWESNG	QPENNYKTTP	PVLDSGSGFF	YSKLTVDKSR
210	220	230	240	250
WQOQNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTLMLLA
260	270	280	290	300
QMRRLSLFSC	LKDRHDFGFP	QEEFGNQFQK	AETIPVLHEM	IQQIFNLFST
310	320	330	340	350
KDSSAAHDET	LIDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSTLAV
360	370	380	390	
RKYFORITLY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE

IFN202:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYCKKVS	NALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL
160	170	180	190	200
TCLVKGFP	YS DIAVEWESNG	QPENNYKTTP	PVLDSGSGFF	YSKLTVDKSR
210	220	230	240	250
WQOQNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTLMLLA
260	270	280	290	300
QMRRLSLFSC	LKDRHDFGFP	QEEFGNQFQK	AETIPVLHEM	IQQIFNLFST
310	320	330	340	350
KDSSAAHDET	LIDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSTLAV
360	370	380	390	
RKYFORITLY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE

FIGURE 5.8

IFN219:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL
160	170	180	190	200
TCLVKGFPYS	DIAVEWESNG	OPENNYKTTP	PVLDSGDSFF	YSKLTVDKSR
210	220	230	240	250
WQQGNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTMLLIA
260	270	280	290	300
QMRRISLFSC	LKDRHDFGPP	QEEFGNQFOK	AETIPVLYEM	IQQITNLFST
310	320	330	340	350
KDSSAAHDET	LLDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSILAV
360	370	380	390	
RKYFORITLY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE

IFN248:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL
160	170	180	190	200
TCLVKGFPYS	DIAVEWESNG	OPENNYKTTP	PVLDSGDSFF	YSKLTVDKSR
210	220	230	240	250
WQQGNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTMLLIA
260	270	280	290	300
QMRRISLFSC	LKDRHDFGPP	QEEFGNQFOK	AETIPVLYEM	IQQIANLFST
310	320	330	340	350
KDSSAAHDET	LLDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSILAV
360	370	380	390	
RKYFORITLY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE

FIGURE 5.9

IFN270:					
10	20	30	40	50	
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD	
60	70	80	90	100	
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN	
110	120	130	140	150	
GKEYCKKSVN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL	
160	170	180	190	200	
TCLVKGFPYS	DIAVEWESNG	QPENNYKTTP	PVLDSGDSFF	YSKLTVDKSR	
210	220	230	240	250	
WQOQNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTMLLLA	
260	270	280	290	300	
QMRRISLFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	TQQIANLFST	
310	320	330	340	350	
KDSSAAHDET	LIDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSNLAV	
360	370	380	390		
RKYFQRITNY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE	

IFN273:					
10	20	30	40	50	
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD	
60	70	80	90	100	
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN	
110	120	130	140	150	
GKEYCKKSVN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL	
160	170	180	190	200	
TCLVKGFPYS	DIAVEWESNG	QPENNYKTTP	PVLDSGDSFF	YSKLTVDKSR	
210	220	230	240	250	
WQOQNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTMLLLA	
260	270	280	290	300	
QMRRISLFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	TQQIANLFST	
310	320	330	340	350	
KDSSAAHDET	LIDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSNLAV	
360	370	380	390		
RKYFQRITNY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE	

FIGURE 5.10

IFN276:					
10	20	30	40	50	
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD	
60	70	80	90	100	
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN	
110	120	130	140	150	
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL	
160	170	180	190	200	
TCLVKGFPYS	DIAVEWESNG	OPENNYKTTT	PVLDSGSGFF	YSKLTVDKSR	
210	220	230	240	250	
WQOQNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTLMLLA	
260	270	280	290	300	
QMRRLSIFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	TOQIANLFST	
310	320	330	340	350	
KDSSAAHDET	LIDKIFYTELY	QQLNDLEACV	IQGVGVTEPT	LMKEDSTLAV	
360	370	380	390		
RKYFORITNY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLSRKE	

IFN306:					
10	20	30	40	50	
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD	
60	70	80	90	100	
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN	
110	120	130	140	150	
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL	
160	170	180	190	200	
TCLVKGFPYS	DIAVEWESNG	OPENNYKTTT	PVLDSGSGFF	YSKLTVDKSR	
210	220	230	240	250	
WQOQNVFSCS	VMHEALHNHY	TOKSLSLSPG	KCDLPQTHSL	GSRRTLMLLA	
260	270	280	290	300	
QMRRLSIFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	IQQIFNLFS	
310	320	330	340	350	
KDSSAAHDET	LIDKIFYTELY	QQLNDLEACV	IQGVGVTEPT	LMKEDSILAV	
360	370	380	390		
RKYFORITLY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLSRKE	

FIGURE 5.11

IFN311:					
10	20	30	40	50	
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD	
60	70	80	90	100	
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN	
110	120	130	140	150	
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL	
160	170	180	190	200	
TCLVKGFYPS	DIAVEWESNG	OPENNYKTTT	PVLDSGSGFF	YSKLTVDKSR	
210	220	230	240	250	
WQOQNVFSCS	VMHEALHNHY	TQKSLSLSPG	KCDLPQTHSL	GSRRTIMLLA	
260	270	280	290	300	
QMRRLQSLFSC	LKDRHDFGFP	QEEFGNQFOK	AETIPVLHEM	TQQIANLFST	
310	320	330	340	350	
KDSSAHDDET	LDDKFYTETY	QQLNDLEACV	IQGVGVTEPT	LMKEDSRLAV	
360	370	380	390		
RKYFORITNY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE	
IFN316:					
10	20	30	40	50	
EPKSSDKTHT	CPPCPAPELL	GGPSVFLFPP	KPKDTLMISR	TPEVTCVVVD	
60	70	80	90	100	
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN	
110	120	130	140	150	
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	PQVYTLPPSR	EEMTKNQVSL	
160	170	180	190	200	
TCLVKGFYPS	DIAVEWESNG	OPENNYKTTT	PVLDSGSGFF	YSKLTVDKSR	
210	220	230	240	250	
WQOQNVFSCS	VMHEALHNHY	TQKSLSLSPG	AGGGSGGGG	SGGGSGCDLP	
260	270	280	290	300	
QTHSLGSRRT	LMLLAQMRQ	SLFSLCKDRH	DFGFPQEEFG	NOFQKAETIP	
310	320	330	340	350	
VLHEMTQQIA	NLFSTKDSSA	AHDETLLDKF	YTELYQQLND	LEACVIQGVG	
360	370	380	390	400	
VTETPLMKED	SRLAVRKYFQ	RITNYLKEKK	YSPCAWEVVR	AEIMRSFSL	
410					
TNLQESLRSK	E				

FIGURE 5.12

IFN120:

10	20	30	40	50
EPKSSDKTHT	CPPCPAPELL	GGPSVFLPPP	KPKDTLMISR	TPEVTCVVVD
60	70	80	90	100
VSHEDPEVKF	NWYVDGVEVH	NAKTKPREEQ	YNSTYRVVSV	LTVLHQDWLN
110	120	130	140	150
GKEYKCKVSN	KALPAPIEKT	ISKAKGQPRE	POVYTLPPSR	EEMTKNOVSL
160	170	180	190	200
TCLVKGFYPS	DIAVEWESNG	OPENNYKTTP	PVLDSGGSFF	YSKLTVDKSR
210	220	230	240	250
WQQGNVFS	VMHEALHNHY	TOKSLSLSPG	AGGGSGGGG	SGGGSGCDLP
260	270	280	290	300
QTHSLGSRRT	LMLLAQMRR	SLFSLCKDRH	DFGFPQEEFG	NQFQKAETIP
310	320	330	340	350
VIHEMIQQIF	NLFSTKQSSA	ANDETLLDKF	YTELYQQQLND	LEACVIQGVG
360	370	380	390	400
VTETPLMKED	SILAVRKYFQ	RITLYLKEKK	YSPCAWEVVR	AEIMRSFSL
410				
TNLQESLR	SK	E		

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(74) Common Representative: **MERCK PATENT GMBH**; Frankfurter Strasse 250, 64293 Darmstadt (DE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: FUSION PROTEINS OF INTERFERON ALPHA MUTEINS WITH IMPROVED PROPERTIES

(57) Abstract: The invention concerns human interferon alpha and in particular modified forms of interferon alpha 2 with improved properties. The improved proteins contain amino acid substitutions at specific positions that confer increased relative activity in biological assays. The invention provides also modified interferon alpha with improved biological activity concomitant with reduced immunogenic potential in the protein. The improved proteins are intended for therapeutic use in the treatment of diseases in humans.



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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP2004/001524

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/21 C07K14/56 A61K38/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/085941 A (MERCK PATENT GMBH ; BAKER MATTHEW (GB); CARTER GRAHAM (GB); HANLON MAR) 31 October 2002 (2002-10-31) cited in the application page 6 - page 18; figure 2 -----	1,4-13
A	WO 97/24137 A (TANOX BIOSYSTEMS INC) 10 July 1997 (1997-07-10) cited in the application page 3 ----- -/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

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Date of the actual completion of the international search

21 December 2004

Date of mailing of the international search report

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Schneider, P

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP2004/001524

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOLLIGER P ET AL: "DIABODIES: SMALL BIVALENT AND BISPECIFIC ANTIBODY FRAGMENTS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 90, 1 July 1993 (1993-07-01), pages 6444-6448, XP002008022 ISSN: 0027-8424 cited in the application page 6448; figure 1 -----	
A	US 6 331 525 B1 (CHIOU HENRY C ET AL) 18 December 2001 (2001-12-18) the whole document -----	
P,A	DATABASE EMBL 14 January 2004 (2004-01-14), XP002311723 Database accession no. AX962744 the whole document	
P,A	& WO 03/104803 A (MERCK PATENT GMBH ; BAKER MATTHEW (GB); CARTER GRAHAM (GB); CARR FRANC) 18 December 2003 (2003-12-18) -----	
E	WO 2004/031352 A (DESJARLAIS JOHN RUDOLPH ; MARSHALL SHANNON ALICIA (US); CHO HO SUNG (U) 15 April 2004 (2004-04-15) page 16; claims 1-37 -----	1-6,9-13
E	WO 2004/022593 A (GUYON THIERRY ; VEGA MANUEL (FR); DRITTANTI LILA (FR); GANTIER RENE (F) 18 March 2004 (2004-03-18) page 79 page 126 -----	1-6,9-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/001524

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claim: 1 and 4 to 13 (all partially)

A modified human interferon alpha 2 (mutein) with amino acid "Q" at position X1 irrespective of any other mutation, with or without linker, a DNA sequence encoding said mutein, a pharmaceutical composition comprising said mutein and its use for the manufacture of a medicament

2. claims: 1, 4 to 13 (all partially)

Same as invention 1 with X2 = "Y" as far as not covered by subject 1

3. claims: 1, 3 to 13 (all partially)

Same as invention 1 with X3 = "T" as far as not covered by subject 2

4. claims: 1, 3 to 13 (all partially)

Same as invention 1 with X4 = "T" or "A" as far as not covered by subject 3

5. claims: 1, 2 and 4 to 13 (all partially)

Same as invention 1 with X5 = "H" as far as not covered by subject 4

6. claims: 1, 4 to 13 (all partially)

Same as invention 1 with X6 = "D" as far as not covered by subject 5

7. claims: 1, 4 to 13 (all partially)

Same as invention 1 with X7 = "N", "T", "P" or "R" as far as not covered by subject 6

8. claims: 1, 2 and 4 to 13 (all partially)

Same as invention 1 with X8 = "T", "H", "D", "S" or "N" as far as not covered by subject 7

9. claims: 1, 4 to 13 (all partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Same as invention 1 with X9 = "S" as far as not covered by
subject 8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP2004/001524

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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